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JAMES L. SMITH and JOHN A. ALFORD
Eastern Utilization Research and Development Division, Agricultural Research Service
United States Department of Agriculture, Beltsville, Maryland 20705

Action of Microorganisms on the Peroxides and Carbonyls of Rancid Fat

SUMMARY—The effects of 26 species of bacteria, molds, and yeasts on the hydroperoxides and monocarbonyls in rancid fat have been determined. All of the cultures were capable of decomposing the hydroperoxides. The activity of microorganisms on the monocarbonyl content of the rancid fat was quite varied and could be divided into: 1) microorganisms which produced large increases in at least two monocarbonyl classes; 2) microorganisms which removed 2,4-dienals; 3) microorganisms which removed 2,4-dienals and 2-enals, and 4) microorganisms which caused decreases in at least two classes of monocarbonyls (without destroying completely any class).

Two microorganisms produced methyl ketones, a monocarbonyl class which does not appear in rancid lard. There appeared to be a relationship between the ability to decompose peroxides strongly and the ability to produce a great increase in the monocarbonyl content. There apparently is no relationship between the ability to decompose peroxides and lipolytic activity. The possible importance of microorganisms in controlling hydroperoxides and monocarbonyls in fats is discussed.

INTRODUCTION

MICROORGANISMS DECOMPOSE HYDROPEROXIDES (Updegraff *et al.*, 1958; Finnerty *et al.*, 1962) and can utilize aldehydes (Racker, 1950; Black, 1951; Seegmiller, 1953; DeMoss, 1954). However, there are no reports concerning the action of microorganisms on rancid fats which contain hydroperoxides and carbonyls. Since carbonyl compounds (formed by decomposition of hydroperoxides) have been found to cause flavor changes in certain foods (Hoffmann, 1962; Day, 1966), we decided to investigate the effect of microorganisms on the peroxides and monocarbonyl content of rancid lard.

METHODS AND MATERIALS

Preparation of rancid lard emulsion

Freshly rendered lard was spread in thin layers in baking dishes and exposed to ultraviolet light at 25°C until the peroxide value was 90–100 meq/kg fat (24–30 hr). Emulsions containing 20% rancid lard were prepared as described previously (Alford *et al.*, 1963), except that the nitrogen atmosphere was eliminated.

Microorganisms and media

The microorganisms utilized are listed in Table 1 along with the conditions of incubation. The cultures were obtained from a number of different sources and were assigned numbers in our collection for ease of identification. These strain designations are given in Table 1. The media used in this study were:

Medium 1—Case peptone, 1.0 g; 1*M* phosphate buffer (pH 7.0), 5 ml; water, 94 ml.

Medium 2—NH_4Cl, 1.0 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.12 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g; $MnSO_4 \cdot H_2O$, 0.01 g; Edamin S (Sheffield), 40.0 g; glucose, 2.5 g (25 ml of a 10% solution was sterilized separately); distilled water, 933 ml.

Medium 3—Bacto tryptose phosphate broth with additional 2% tryptose.

Medium 4—Case peptone, 2.0 g; Difco yeast extract, 0.1 g; 1.0*M* phosphate buffer (pH 7.0), 5 ml; distilled water, 93 ml.

Medium 5—Case peptone, 1.0 g; Difco yeast extract, 0.1 g; 1.0*M* phosphate buffer (pH 6.0), 5 ml; distilled water, 94 ml.

Medium 6—Difco veal infusion broth.

Medium 7—Case peptone, 1.0 g; Difco yeast extract, 0.1 g; 1.0*M* phosphate buffer (pH 7.0), 5 ml; distilled water 94 ml.

Medium 8—Difco nutrient broth plus 3% glucose (sterilized separately as a 10% solution).

Medium 9—Difco nutrient broth.

Medium 10—Difco tryptose phosphate broth.

Medium 11—BBL APT broth.

Wherever phosphate buffer was used it was equimolar Na:K. All media were sterilized by autoclaving at 121°C for 15 min. For static cultures, 500 ml of medium were dispensed into 3-L, low-form culture flasks. For shaken cultures, 125 ml of medium were dispensed into 1-L Erlenmeyer flasks; after growth had occurred, 500 ml of growth culture were dispensed into sterile 3-L, low-form culture flasks.

Action of microorganisms on rancid fat

When lipase production had reached its peak (or when cell growth was near the maximum if no lipase was produced), non-sterile rancid lard emulsion was added to the cultures so that the final concentration of lard was 3%. All culture-lard emulsion mixtures were incubated at the appropriate temperatures (Table 1) as stationary cultures until 80–100% of the peroxides had disappeared, or for five days. Uninoculated medium-lard emulsion mixtures were utilized as controls and were kept refrigerated at 4–5°C until needed. The rancid lard emulsion was not sterilized because heat changed the monocarbonyl pattern drastically (Gaddis *et al.*, 1959a).

Assay of lipase

Lipase assays were determined as previously described (Alford *et al.*, 1963) with incubation at 35°C for one hour. All cultures were assayed at pH 7.0 except *Candida lipolytica, Aspergillus niger, Rhizopus oligosporus,* and *Thamnidium elegans,* which were assayed at pH 6.0.

Table 1. Microorganisms employed and methods of producing cells for study of effect on rancid lard.

Microorganisms	Medium used[1]	Incubation of culture before the addition of lard emulsion
		Static cultures
Pseudomonas fragi (43)	1	20°C, 3 days
Pseudomonas species (92)	1	20°C, 4 days
Pseudomonas ovalis (36)	1	20°C, 3 days
Streptomyces species (280 & 281)	9	25°C, 10 days
Streptococcus lactis (263)	11	20°C, 24 hours
Streptococcus cremoris (264)	11	20°C, 24 hours
Lactobacillus casei (266)	11	25°C, 24 hours
Pediococcus cervisiae (270)	11	25°C, 24 hours
Leuconostoc citrovorum (267)	11	25°C, 24 hours
Leuconostoc dextranicum (268)	11	25°C, 24 hours
Leuconostoc mesenteroides (269)	11	25°C, 24 hours
Candida lipolytica (181)	5	20°C, 4 days
Aspergillus niger (172)	4	35°C, 3 days
Aspergillus flavus (88)	4	25°C, 5 days
Rhizopus oligosporus (173)	5	20°C, 5 days
Thamnidium elegans (195)	5	20°C, 4 days
Penicillium roqueforti (174)	7	30°C, 10 days
Geotrichum candidum (165)	2	20°C, 4 days
		Shaker cultures
Serratia marcescens (279)	6	25°C, 200 rpm, 24 hours
Escherichia coli (107)	6	25°C, 100 rpm, 24 hours
Staphylococcus aureus (63)	3	35°C, 400 rpm, 24 hours
Staphylococcus aureus (66)	10	35°C, 400 rpm, 24 hours
Micrococcus cryophilus (90)	10	20°C, 200 rpm, 24 hours
Micrococcus freudenreichii (115)	10	20°C, 200 rpm, 24 hours
Sarcina lutea (112)	10	25°C, 200 rpm, 24 hours
Bacillus cereus (283 & 284)	6	25°C, 100 rpm, 24 hours
Hansenula anomala (282)	8	25°C, 200 rpm, 24 hours

[1] The composition of the various media is given in the text.

Peroxide determination

The peroxide value was determined by the cold method of Lea (1952).

Monocarbonyl determination

At the end of the incubation period, the culture-lard emulsion mixtures were centrifuged and the fat was extracted from the supernatant with petroleum ether (40–60°C b.p. range). The monocarbonyl fraction was isolated by column chromatography as described by Schwartz *et al.* (1962, 1963), and monocarbonyls were further separated into classes by the chromatographic techniques of Gaddis *et al.* (1959b). The classes were identified by their R_f values on paper and by their absorption maxima utilizing a Cary recording spectrophotometer. To make it possible to calculate concentrations on a molar basis, an average molecular weight of 1000 was assumed for the lard.

RESULTS

THE MONOCARBONYL COMPOUNDS isolated from rancid lard consist of three classes: 2,4-dienals, 2-enals, and n-alkanals. Although the microorganisms studied in this investigation varied widely in the extent of their action on these monocarbonyls, the activity of any given culture was consistent and repeatable. Each value shown in Tables 2–6 is from a representative run. Every experiment was repeated at least once and with most organisms three to six times. Although initial values on the rancid lard varied among runs, the percentage variation among runs for the same microorganism was always within 10 to 20%. On the basis of this activity the microorganisms could be divided into five groups.

Group 1 produced relatively large increases in at least two of the monocarbonyl classes. The data for this group are recorded in Table 2. The activity of *Aspergillus flavus* in removing peroxides coupled with the sharp increases in 2,4-dienals, alkanals, and methyl ketones made it the most active culture in monocarbonyl production. *Streptococcus lactis* was the only culture of the group which caused a large increase in all three of the main monocarbonyl classes.

The complete destruction of the 2,4-dienal class of the monocarbonyl fraction of rancid lard was the most significant action of the microorganisms that belong to Group 2. At the present time, the products of the decomposition are unknown. The data for the group are presented in Table 3. Strong lipolytic and peroxide decomposing activity was shown by *Pseudomonas fragi*; other members of the group were non-lipolytic and possessed weak peroxide-destroying capacity. *P. fragi* also produced a large quantity of methyl ketones. It and *A. flavus* were the only two cultures of the 29 examined which produced this type of monocarbonyl.

The microorganisms that decomposed completely both the 2,4-dienals and 2-enals were placed together in Group 3, and the data for the group are presented in Table 4. Cultures of *Staphylococcus aureus, Rhizopus oligosporus,* and *Geotrichum candidum* were active lipase producers and a majority of the organisms in the group possessed a strong capacity to decompose peroxides. Eight of 10 orga-

Table 2. Microorganisms producing relatively large increases in at least two classes of monocarbonyls in rancid fat (Group 1).

Microorganisms	Lipase activity when fat added μeq FFA/hr	Peroxide value		Monocarbonyls[1]			
		Start meq/kg fat	End meq/kg fat	2,4-Dienals	2-Enals	Alkanals	Methyl ketones
A. flavus (88)	153.2	75.5	0.0 (3 days)	6.1	3.9	48.1	30.9
Control	......	75.5	74.3	1.5	3.6	21.3	0.0
S. lactis (263)	6.4	81.4	45.1 (5 days)	6.8	8.8	50.6	0.0
Control	......	81.4	75.8	1.6	3.8	30.4	0.0
P. cerevisiae (270)	5.0	80.8	59.3 (5 days)	3.3	5.3	29.1	0.0
Control	......	80.8	74.7	1.4	2.9	26.2	0.0
L. dextranicum (268)	8.6	77.4	29.8 (5 days)	3.6	6.0	19.9	0.0
Control	......	77.4	73.0	1.6	3.5	20.2	0.0
M. freudenreichii (115)	0.0	75.0	50.5 (5 days)	9.8	7.7	19.9	0.0
Control	......	75.0	76.4	1.4	5.1	38.2	0.0

[1] Expressed as $\mu M/10^4$ μM fat.

Table 3. Microorganisms which completely remove the 2,4-dienals from rancid fat (Group 2).

Microorganisms	Lipase activity when fat added μeq FFA/hr	Peroxide value		Monocarbonyls[1]			
		Start meq/kg fat	End meq/kg fat	2,4-Dienals	2-Enals	Alkanals	Methyl ketones
P. ovalis (36)	0.0	73.9	62.7 (5 days)	0.0	5.1	35.2	0.0
Control	......	73.9	76.8	2.2	5.2	39.2	0.0
Pseudomonas sp. (92)	10.4	78.2	65.7 (5 days)	0.0	2.5	23.2	0.0
Control	......	78.2	74.0	1.6	3.6	22.3	0.0
P. fragi (43)	111.1	82.1	25.6 (5 days)	0.0	7.7	45.4	36.5
Control	......	82.1	83.8	1.5	6.7	34.9	0.0
B. cereus (284)	9.0	77.6	57.2 (5 days)	0.0	2.0	16.9	0.0
Control	......	77.6	76.5	1.5	3.9	44.4	0.0
B. cereus (283)	10.0	77.6	55.6 (5 days)	0.0	2.5	23.7	0.0
Control	......	77.6	76.5	1.5	3.9	44.4	0.0

[1] Expressed as $\mu M/10^4$ μM fat.

Table 4. Microorganisms which completely removed 2,4-dienals and 2-enals from rancid lard (Group 3).

Microorganisms	Lipase activity when fat added μeq FFA/hr	Peroxide value		Monocarbonyls[1]		
		Start meq/kg fat	End meq/kg fat	2,4-Dienals	2-Enals	Alkanals
E. coli (107)	0.0	82.8	11.7 (5 days)	0.0	0.0	22.4
Control	......	82.8	80.8	1.4	3.2	24.6
S. marcescens (279)	22.4	71.8	33.9 (5 days)	0.0	0.0	51.6
Control	......	71.8	64.5	1.2	3.4	20.1
M. cryophilus (90)	18.0	76.5	9.8 (5 days)	0.0	0.0	30.2
Control	......	76.5	75.1	1.5	3.5	40.0
S. aureus (63)	217.0	77.6	0.0 (3 days)	0.0	0.0	46.1
Control	......	77.6	66.9	1.1	2.7	26.4
S. lutea (112)	3.0	75.0	8.4 (5 days)	0.0	0.0	88.1
Control	......	75.0	76.5	1.4	5.1	38.2
S. aureus (66)	310.4	73.7	8.1 (3 days)	0.0	0.0	58.3
Control	......	73.7	75.1	0.9	2.3	23.4
P. roqueforti (174)	2.1	68.3	27.9 (5 days)	0.0	0.0	60.4
Control	......	68.3	68.3	2.5	5.8	40.8
A. niger (172)	5.1	110.0	20.7 (3 days)	0.0	0.0	41.1
Control	......	110.0	106.9	1.6	4.7	24.9
R. oligosporus (173)	96.8	118.1	23.4 (4 days)	0.0	0.0	71.4
Control	......	118.1	115.9	1.7	5.7	33.6
G. candidum (165)	115.1	105.4	0.0 (3 days)	0.0	0.0	58.3
Control	......	105.4	97.4	2.0	4.3	18.7

[1] Expressed as $\mu M/10^4$ μM fat.

Table 5. Microorganisms causing decreases in at least two classes of monocarbonyls but which do not completely remove any class (Group 4).

Microorganisms	Lipase activity when fat added µeq FFA/hr	Peroxide value Start meq/kg fat	Peroxide value End meq/kg fat	Monocarbonyls[1] 2,4-Dienals	Monocarbonyls[1] 2-Enals	Monocarbonyls[1] Alkanals
C. lipolytica (181)	51.9	95.1	11.8 (3 days)	0.8	2.2	32.3
Control	......	95.1	94.9	3.4	6.6	39.8
Streptomyces sp. (281)	2.2	83.4	65.5 (5 days)	0.6	1.5	12.2
Control	......	83.4	80.0	1.7	3.7	29.8
Streptomyces sp. (280)	2.2	83.4	53.7 (5 days)	0.5	1.5	13.9
Control	......	83.4	80.0	1.7	3.7	29.8
H. anomala (282)	0.0	64.7	40.3 (5 days)	1.9	2.6	12.8
Control	......	64.7	68.8	1.3	3.2	20.0
L. casei (266)	0.0	79.1	37.1 (5 days)	1.1	3.3	24.8
Control	......	79.1	77.9	1.9	4.6	34.1

[1] Expressed as $\mu M/10^4$ μM fat.

nisms produced a large increase in the alkanal content, thus leading to an increase in the total monocarbonyl concentration.

Those cultures which caused a decrease in at least two monocarbonyl classes, but were not sufficiently active to remove any one of them completely, are shown in Table 5.

The remaining cultures examined have been arbitrarily combined in a single group that is characterized by relatively weak ability to alter the monocarbonyl content of rancid lard (Table 6). Although the slight increases or decreases were consistent in repeated trials, they did not have a great effect on the total monocarbonyl content of the rancid fat, and the activity in reducing the peroxide level was moderate to weak.

It was obvious from observing the 2,4-dinitrophenylhydrazone bands that developed on the Seasorb and alumina columns that most of the microorganisms utilized in this study caused changes in several types of polar carbonyls present in the rancid fat in addition to the changes produced in the monocarbonyl fraction. Both strains of *S. aureus* and *A. flavus* destroyed ketoglycerides so that no ketoglyceride band was visible on the alumina columns. None of the other cultures had any discernible effect on the ketoglyceride band. With most of the microorganisms utilized, the odor and taste of the rancid lard were changed so that the typical organoleptic response to rancid lard was not obtained.

DISCUSSION

Hydroperoxides are decomposed by both animal tissues and microbial cells. A variety of microorganisms and animal tissues decompose t-butyl, p-menthane, and cumeme hydroperozides; however, none of the products were identified (Updegraff *et al.*, 1958). *Micrococcus cerificans* metabolizes 1-alkyl hydroperoxides (C_{12}, C_{14}, C_{16}, C_{18}); 1-octyldecyl hydroperoxide was transformed into stearyl stearate (Finnerty *et al.*, 1962). Although the hydroperides present in rancid fat were decomposed by almost all of the microorganisms we investigated, the differences in the extent of attack ranged from a small percentage decrease by *Leuconostoc mesenteroides* to 85–100% destruction by *S. aureus*, *A. flavus*, and *Candida lipolytica*.

Corresponding increases in monocarbonyls were not found, however. *A. flavus* produced an increase in all monocarbonyl fractions including methyl ketones, while *C. lipolytica* decreased the three main classes of monocarbonyls. There does not appear to be any relationship between strong lipolytic activity and the ability to strongly decompose peroxides; for example, *Escherichia coli*, *Micrococcus cryophilus*, and *Aspergillus niger* had little lipase activity, yet 80% or more of the hydroperoxides were decomposed.

The development of a green flavor defect in mixed strain lactic starter cultures is a serious problem encountered in the manufacture of cultured buttermilk and sour cream,

Table 6. Microorganisms with weak activity relative to monocarbonyls (Group 5).

Microorganisms	Lipase activity when fat added µeq FFA/hr	Peroxide value Start meq/kg fat	Peroxide value End meq/kg fat	Monocarbonyls[1] 2,4-Dienals	Monocarbonyls[1] 2-Enals	Monocarbonyls[1] Alkanals
S. cremoris (264)	8.0	80.8	74.7 (5 days)	1.6	4.0	27.6
Control	......	80.8	74.7	1.4	2.9	26.2
T. elegans (195)	35.8	115.0	43.1 (5 days)	2.9	5.3	27.6
Control	......	115.0	121.0	2.2	6.8	27.1
L. mesenteroides (269)	5.4	79.1	76.0 (5 days)	0.7	4.1	33.9
Control	......	79.1	77.9	1.9	4.6	34.1
L. citrovorum (267)	5.6	79.1	58.0 (5 days)	0.8	4.0	30.2
Control	......	79.1	77.9	1.9	4.6	34.1

[1] Expressed as $\mu M/10^4$ μM fat.

and acetaldehyde has been shown to be responsible for the undesirable flavor (Lindsay *et al.,* 1965). Certain *Leuconostoc* species which can decompose acetaldehyde added to the starter eliminated this defect (Keenan *et al.,* 1966a; Keenan *et al.,* 1966b). Short chain aliphatic aldehydes are oxidized to the corresponding acids by dehydrogenases isolated from yeast (Black, 1951; Seegmiller, 1953), and the existence of alcohol dehydrogenases capable of reducing short chain aldehydes to the corresponding alcohols has been reported (Racker, 1950; DeMoss, 1954).

The ability of microorganisms to selectively attack specific types of compounds is supported by the data reported here. The apparently wide range of possible effects that can be obtained by judicious selection of cultures indicates that it might be feasible to selectively produce or remove volatile carbonyl compounds in fats or fat-containing foods.

The production of methyl ketones from fat by microorganisms has been well established (Hawke, 1966). Lipolytic fungi growing on fats containing low molecular weight triglycerides liberate fatty acids which are converted to β-keto acids and then decarboxylated to form the methyl ketones. Both of the cultures found to produce methyl ketones in our study were lipolytic, and a similar route to ketone production may have occurred.

It also has been shown that methyl ketones can be produced from alkanes by *Pseudomonas methanica* (Leadbetter *et al.,* 1960) and by *Myobacterium smegmatis* (Lukins *et al.,* 1963). Alkanes have been shown to be products of rancidity (Evans, 1961) and, thus, methyl ketone production by *P. fragi* and *A. flavus* may have been derived from alkanes rather than products of lipase activity.

The use of microorganisms to remove monocarbonyls and peroxides from rancid fat and to delay the onset of autoxidation in fresh lard and other fats is being investigated. Although no attempt was made to determine the similarity of activity within taxonomic groups, three species within the genus *Pseudomonas* were similar as were two strains of *S. aureus* and two of *B. cereus.* Further investigation of this activity may provide additional criteria for their differentiation.

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